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# The zinc binding receptor GPR39 interacts with 5-HT$_{1A}$ and GalR$_1$ to form dynamic heteroreceptor complexes with signaling diversity

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ABSTRACT

GPR39 is a class A G protein-coupled receptor involved in zinc binding and glucose homeostasis regulation, among other physiological processes. GPR39 was originally thought to be the receptor for obestatin peptide but this view has been challenged. However, activation of this receptor by zinc has been clearly established. Recent studies suggest that low GPR39 expression, due to deficient zinc levels, is involved in major depressive disorder. We have previously reported that zinc can alter receptor–receptor interactions and favor specific receptor interactions. In order to unravel the effect of zinc on specific G protein-coupled receptor association processes, we have performed FRET and co-immunopurification studies with GPR39 and 5-HT$_{1A}$ and GalR$_1$ which have been shown to dimerize. Our results suggest that zinc can modulate the formation of specific 5-HT$_{1A}$-GPR39 and GalR$_1$-5-HT$_{1A}$-GPR39 heteroreceptor complexes under our experimental conditions.
We have analyzed the differences in signaling between the mono-homomeric receptors 5-HT$_{1A}$, GalR$_1$ and GPR39 and the heteroreceptor complexes between them Our results show that the GPR39-5-HT$_{1A}$ heterocomplex has additive functionalities when compared to the monomeric–homomeric receptors upon receptor activation. In addition, the heterocomplex including also GalR$_1$ shows a different behavior, upon exposure to the same agonists. Furthermore, these processes appear to be regulated by zinc. These findings provide a rationale for the antidepressive effect widely described for zinc because pro-depressive heterocomplexes are predominant at low zinc concentration levels.



## 1. Introduction

GPR39 is a member of the rhodopsin family of class A G protein-coupled receptors (GPCRs) [1]. It was first cloned in 1997 [2] and described as two isoforms; the full-length receptor (GPR39-1a) and a truncated transmembrane domain isoform (termed GPR39-1b). Until now, however, its specific ligand is still a matter of debate. For instance, it was initially thought to be the receptor for the obestatin peptide [3], but no clear reproducible data have been reported to confirm this proposal [4,5]. Furthermore, activation of the receptor by zinc, which would function as an agonist only for the full-length GPR39-1a isoform [6], has been also widely described [4,5].

A wide variety of physiological roles have been described for this receptor, like regulation of gastrointestinal and metabolic functions [7] and apoptosis protection [8], or its involvement in certain disorders like obesity type 2 associated diabetes mellitus [9], pancreatic islet dysfunction [10], cancer [11] and depression [12,13]. In this work we have focused on the potential involvement of GPR39 in depression. There is consensus on the fact that GPR39 can be activated by zinc and there is growing evidence that this interaction may be involved in the pathophysiology of depression [12,13]. One of the main current hypotheses is that a zinc deficient environment can result in decreased expression of GPR39 and this may be linked to depressive behavior. The association of GPR39, zinc and depression is appealing and may be parallel to our observed effect of zinc on 5-hydroxytriptamine 1A receptor (5-HT$_{1A}$)-galanin receptor 1 (GalR$_1$) heteromer [14]. The current study was based on a previous hypothesis, which suggested that 5-HT$_{1A}$-GalR$_1$ heteromer is involved in major depressive disorder [15], and it was concluded that zinc may disrupt the 5-HT$_{1A}$-GalR$_1$ heteromer through its specific interaction with 5-HT$_{1A}$ and therefore could favor a healthy phenotype [14]. These data could help explain the benefits of zinc supplementation (used in depression treatment) that have been widely recognized [16,17]. Thus, the main scheme of this working hypothesis is based on the complex interplay among the GPCR-zinc-depression triad. It is plausible that the three aforementioned receptors, GPR39, 5-HT$_{1A}$ and GalR$_1$, can be interlinked and play a complementary role in the molecular mechanism of depression. This is supported by the concept that GPCRs can form homo and heteroreceptor complexes, which may act as new signaling units [18–20]. 5-HT$_{1A}$ forms heteroreceptor

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complexes with different GPCRs [15,21]. Therefore, the hypothesis appears valid that 5-$HT_{1A}$, GPR39 and $GalR_1$ can arrange into different oligomerization states where 5-$HT_{1A}$ is the hub receptor.

We have experimentally tested the existence of the proposed interactions in order to gain new insights into this question. First, we have analyzed the potential 5-$HT_{1A}$-GPR39 interactions, because 5-$HT_{1A}$-$GalR_1$ heteromers had been already described [14,15]. We have validated that there is co-localization between the two receptors by means of fluorescence microscopy. By using a complementary approach combining FRET (Förster resonance energy transfer) and co-immunopurification, we validate the interaction of 5-$HT_{1A}$ with GPR39 and $GalR_1$ together or separately. Finally, we have used a gene reporter assay to unravel the possible signaling differences between mono-homomeric receptors and heteroreceptor complexes in their corresponding signal transduction pathways. We have analyzed 5-$HT_{1A}$-GPR39 and 5-$HT_{1A}$-GPR39-$GalR_1$ heteroreceptor complexes activities by measuring its capacity to induce the transduction over the serum response element (SRE) and nuclear factor κβ response element (NFκβ-RE). Our results show that GPR39-5-$HT_{1A}$ heterocomplex has additive functionalities when compared to the monomeric–homomeric receptors upon their activation. In addition, the complex including also $GalR_1$ shows a different behavior upon exposure to the same agonists. These results could reflect that both oligomerization forms could be present at the cell membrane and that the presence of one or another would depend on a specific regulation mechanism that may be modulated by zinc.

## 2. Materials and methods

### 2.1. Plasmid constructs

Rho-1D4 tag is an amino acid sequence corresponding to an epitope located in the C-terminal tail of the bovine visual GPCR rhodopsin which is specifically recognized by the monoclonal Rho-1D4 antibody. This tag was added by inverse PCR to the expression vectors pCDNA3.1 containing either $GalR_1$, or 5-$HT_{1A}$ or GPR39 genes (Missouri S&T cDNA Resource $^{Center}$, USA). pGPR39-EYFP and phr$GFP_2$-5$HT_{1A}$ were obtained by subcloning GPR39 into pEYFP-N3 and hr$GFP_2$ into pCDNA3.1–5-$HT_{1A}$ vectors respectively. Cloning was performed using in-fusion® HD Cloning Kit (Clontech, France) according to the manufacturer's protocol. p5-$HT_{1A}$-ECFP and p$GalR_1$-EYFP constructs were obtained as described previously [15]. The pECFP-EYFP construct was obtained by subcloning PCR-amplified EYFP into the pECFP empty vector using traditional cloning. The used primers are listed below:

pCDNA3.1-$GalR_1$-1D4:

Fw: 5′ACGGAGACGAGCCAGGTGGCCCCGGCCTGACTCGAGTCTAGA GGGCC 3′.

Rv: 5′GGCCGGGGCCACCTGGCTCGTCTCCGTCACATGAGTACAATT GGTTG 3′

pCDNA3.1-5-$HT_{1A}$-1D4:

Fw: 5′ACGGAGACGAGCCAGGTGGCCCCGGCCTGACTCGATAGAG GGCCCG 3′.

Rv: 5′ GGCCGGGGCCACCTGGCTCGTCTCCGTCTGGCGGCAGAACTT ACACT 3′.

phr$GFP_2$-5$HT_{1A}$.

Fw: 5′ ACCGAGCTCGGATCCATGGTGAGCAAGCAGATCCTG 3′.

Rv: 5′ ATCCATGGTGGATCCCACCCACTCGTGCAGGCTGCC 3′.

pGPR39-EYFP.

Fw: 5′ CTCGAGCTCAAGCTTATGGCTTCACCCAGCCTCCC 3′.

Rv: 5′ GCGACCGGTGGATCCGCAACTTCATGCTCCTGAAAACC 3′.

The resulting constructs were sequenced to check both appropriate cloning and unspecific mutation absence (Stabvida, Portugal).

### 2.2. Immunofluorescence microscopy

HEK-293S GnTi$^-$ cells (EACC, UK) were seeded onto glass coverslips for 24 h and transfected with pGPR39-EYFP together with p5$HT_{1A}$-1D4 using polyethylenimine (PEI) (Polysciences, Germany). 24 h after transfection, cells were washed with phosphate buffer saline (PBS) (Sigma-Aldrich, Spain) and fixed with formalin 3.7% (Sigma-Aldrich, Spain) for 20 min. After washing twice with PBS, cells were permeabilized and blocked with blocking buffer (5% skimmed milk in Tris buffer saline (TBS) during 1 h at room temperature (RT). Then, cells were incubated first with the primary antibody, Rho-1D4 (dilution 1/2000 from stock) for 30 min at RT. Then, after three washes with TBS buffer containing 0.1% tween-20 (Sigma-Aldrich, Spain), the samples were incubated with anti-EYFP (dilution 1/200 from stock) (MBL, USA) overnight. Subsequently, secondary antibody incubation was performed for 1 h with anti-mouse-FITC (Sigma-Aldrich, Spain), three washing steps as described before and 1 h more with anti-rabbit-Alexa Fluor® 555 (Life technologies, USA). Finally, coverslips were mounted using Vectashield mounting medium with DAPI (Vector Labs, UK). Representative cell images were taken by inverted fluorescence microscopy using an Nikon Eclipse Ti microscope (Isaza, Spain) with a 100× objective.

### 2.3. Co-immunopurification

The constructs encoding 5-$HT_{1A}$-1D4 and GPR39-EYFP were transiently transfected into HEK-293S GnTi$^-$ cells using PEI for heterodimer presence analysis, and the same pair together with $GalR_1$ for trimer analysis. Equivalent quantities of each construct were used in both transfections. The HEK-293S GnTi$^-$ cell line was used because it provides homogeneous glycosylation and allows a better visualization of the protein bands in electrophoretic gels. Cells were routinely grown in DMEM-F12 media (Labclinics, Spain) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (10,000 u/ml) and 1% L-glutamine (200 mM) (Sigma-Aldrich, Spain) at 37 °C and 5% $CO_2$ atmosphere. 48 h after transfection, cells were treated with 10 mM dimethyl apimidate (Sigma-Aldrich, Spain), pH 8–9, in non-supplemented DMEM-F12 media for 1 h at RT. Then cells were harvested and pelleted at 4000 g during 20 min. The pellet was then solubilized in 10 ml per harvested plate of MES buffer containing 2% Triton X-100 and 100 μM of phenylmethylsulfonyl fluoride at 4 °C during 1 h. Solubilized cells were centrifuged, at 35,000 rpm, for 35 min at 4 °C and the supernatant was incubated by gently nutating with CNBr-activated Sepharose 4B Fast Flow (GE healthcare, Spain) coupled to the Rho-1D4 antibody (Cell Essentials, US) during 2.5 h. The sepharose beads were recovered and washed with 50 mM MES buffer, pH 6.0 (Sigma-Aldrich, Spain) containing 0.05% dodecyl maltoside (DM) (Anatrace, UK) for five times. Elution was performed during 3 h with 50 mM MES buffer pH 6.0 containing 0.05% DM and 0.5 mM Rho-1D4 9-mer peptide (TETSQVAPA) (Unitat de Tècniques Separatives i Síntesi de Pèptids, Universitat de Barcelona, Spain).

### 2.4. Immunoblot analysis of heterocomplexes

Eluted samples were mixed with denaturing loading buffer and subjected to SDS-PAGE (3–14%). The gel was run for 40 min at 250 V in Laemmli running buffer and using SDS7B2 pre-stained marker (Sigma-Aldrich, Spain) as a protein standard. The proteins were subsequently transferred onto a nitrocellulose membrane (Sigma-Aldrich, Spain) and blocked with blocking buffer. The membrane was incubated with the following pairs of antibodies, in three rounds separated by antibody stripping using Restore Western blot Stripping Buffer (Thermo Scientific, France). The first immunoblot was performed using anti-EYFP antibody as primary antibody (1:100 dilution from stock) and anti-rabbit-IgG-HRP as secondary antibody (1:5000 dilution from stock). The second one was performed using anti-$GalR_1$ as primary antibody (1:100 dilution from stock) and anti-goat-IgG HRP (1:5000 dilution from stock) as secondary antibody. The third immunoblot was performed using Rho-1D4 (1:1000 dilution from stock) as primary antibody and anti-mouse-IgG-HRP as secondary antibody. All secondary

antibodies and anti-$GalR_1$ were purchased from Santa Cruz Biotechnology, USA. Blots were developed using substrate WesternBright ECL (Advansta, USA).

### 2.5. FRET experiments

#### 2.5.1. 5-$HT_{1A}$-GPR39 complex detection

HEK-293S GnTi$^-$ cells were transiently co-transfected with constructs corresponding to $hrGFP_2$-5-$HT_{1A}$ acting as donor and GPR39-EYFP acting as acceptor. $hrGFP_2$-5-$HT_{1A}$ DNA amount was kept constant at 1 μg in all transfections whereas GPR39-EYFP was gradually increased as follows: 0 μg, 0.5 μg, 1 μg, 1.5 μg, 2 μg, 3 μg and 4 μg. 48 h after transfection, cells were harvested and resuspended in PBS containing 0.1 $g \cdot l^{-1}$ $CaCl_2$, 0.1 $g \cdot l^{-1}$ $MgCl_2$ and 0.1 $g \cdot l^{-1}$ D-glucose (Sigma-Aldrich, Spain). Then, cells were seeded onto 96-well black plates. Cell concentration was adjusted, by determining protein concentration with a BCA kit (Sigma-Aldrich, Spain), to 1 mg/ml. Finally, FRET signals were measured using an Infinite M200 reader (Tecan, Switzerland). Fluorescence was measured at three different conditions: 1 — Ex = 485 nm and Em = 516 nm, for $hrGFP_2$ signal detection; 2 — Ex = 515 nm and Em = 546 nm for EYFP signal detection; 3 — Ex = 485 nm and Em = 546 nm for FRET signal detection. Mock transfected cells were used for background subtraction. FRET between the two fluorophores was determined essentially as previously described [22].

#### 2.5.2. $GalR_1$-5-$HT_{1A}$-GPR39 complex detection

To carry out this experiment we used the following vectors: p5$HT_{1A}$-ECFP as donor, p$GalR_1$-EYFP as acceptor and pECFP-EYFP as a negative control. Cells were transfected in six well plates using the following conditions: donor alone, donor and acceptor (1:1 ratio) and negative control. Same transfections were repeated as described but also adding pCDNA3.1-GPR39 at equivalent concentrations. FRET spectra were recorded using an Infinite M200 reader. Excitation wavelength was set at 420 nm and emission wavelength range was set from 455 nm to 560 nm. Data was smoothed and normalized using Peakfit software (Systat Software, USA).

### 2.6. Luciferase reporter gene assay

We used Bright-Glo™ Luciferase Assay System (Promega, Spain) to detect receptor activation. Reporter gene vectors containing luciferase transcriptionally driven by response elements (pGL4.33 and pGL4.32) were used in these experiments. Cells were co-transfected, in each case, with the corresponding reporter vector and receptors, at equivalent DNA amounts, using 6 well plates. 24 h after transfection, cells were transferred onto 96 well white plates (Perkin Elmer, USA) in starving media (culture media without FBS). Cells were subsequently treated during 4 h, after additional 24 h, with the appropriate ligand concentration in Optimem (Life Technologies, USA). 8-OH-DPAT at a concentration of 100 μM was used as 5-$HT_{1A}$ agonist, 100 μM $ZnCl_2$ as GPR39 agonist and 1 μM gal (1–29) as $GalR_1$ agonist. Luciferase activity was determined according to the manufacturer's protocol with an Infinity M200 reader, using standard luminescence reading conditions. Experiments were performed in triplicate, and four replicates were measured in each experiment. Statistical analyses were performed by one tailed paired t-test with GraphPad Prism (GraphPad Software Inc., USA). A p value of 0.05 and lower was considered significant.

## 3. Results

### 3.1. Immunofluorescence microscopy

One of the main objectives of this work was to determine the potential formation of a 5-$HT_{1A}$ and GPR39 heteroreceptor complexes in cellular model by means of FRET and co-immunopurification assays. To start with, first we determined if the C-terminal tagged 5-$HT_{1A}$-1D4 and GPR39-EYFP receptors co-localized to the same cell compartment. Double immunolabeling of the tagged 5-$HT_{1A}$-1D4 receptor with anti-1D4/FITC and the tagged GPR39-EYFP with the anti-EYFP/Alexa-Fluor555 antibodies showed that both receptors share a similar distributional pattern and significantly co-localized in the membrane cell surface and reticular compartments (Fig. 1A–F).

### 3.2. Heterodimerization between 5-$HT_{1A}$ and GPR39 measured by FRET

We tested the possible heteromer formation between both receptors by means of the biophysical technique FRET spectroscopy. Using a saturation assay we assessed specific FRET between the two receptors, in cells co-expressing a constant amount of $hrGFP_2$-5-$HT_{1A}$ (humanized green fluorescent protein 2 fused to the C-terminal tail of the 5-$HT_{1A}$) (donor) and an increasing concentration of GPR39-EYFP (GPR39 receptor fused to enhanced yellow fluorescent protein) (acceptor). For unspecific FRET measurements, two populations of cells were independently transfected with a single construct and subsequently mixed. Our data can be fit to a hyperbolic curve with an asymptote tending to the maximal FRET value (Fig. 1G), validating the specific interaction between the two receptors. In contrast, unspecific FRET measurements resulted in a linear relationship between n-FRET and acceptor-to-donor

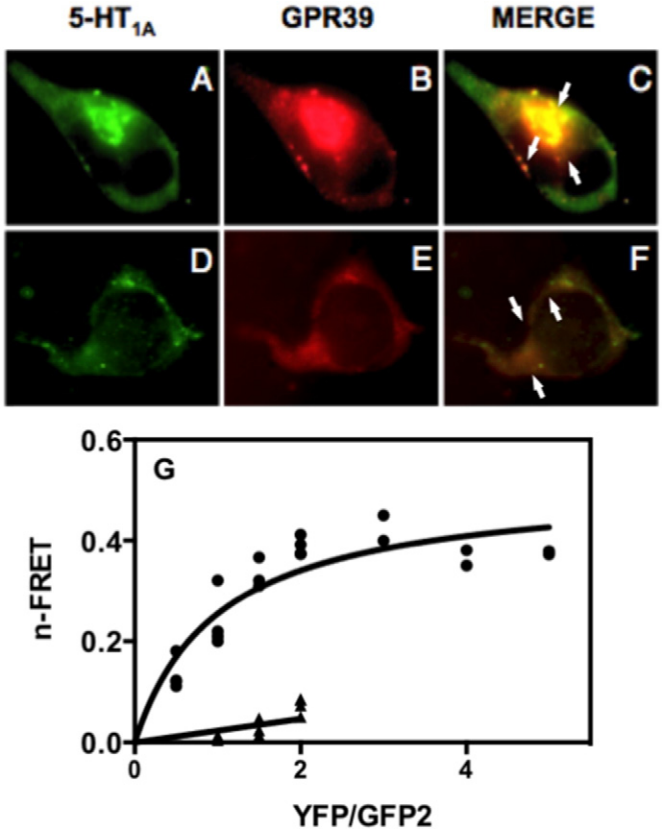


**Fig. 1.** 5-$HT_{1A}$-GPR39 heterodimerization. (A–F) Co-localization of 5-$HT_{1A}$ and GPR39 expressed in HEK293S GnTi$^-$ cells. 5-$HT_{1A}$ corresponds to the green signal (A and D) and GPR39 to red signal (B and E). Green and red signal overlap is shown by the yellowish color giving the area of receptor co-localization (C and F). Pictures show the area of colocalization (white arrows) of the two receptors, which in F is clearly demonstrated along the cell membrane and endoplasmic reticulum. (G) FRET saturation curve between 5-$HT_{1A}$ and GPR39. N-FRET (normalized FRET values) are represented against the acceptor to donor ratio. Specific FRET, corresponding to the co-transfection of $hrGFP_2$-5-$HT_{1A}$ and GPR39-EYFP, (●) is indicated from the saturation profile of the curve. In contrast, unspecific FRET (▲) measurements, corresponding to mixed populations of cells expressing single receptors, exhibit a linear tendency corresponding to a non-specific interaction. Data corresponds to the average of three independent experiments. Data reflect specific interactions between the two receptors.

ratios reflecting lack of interaction between the two mixed populations of cells expressing the individual receptors. Taken together, these results demonstrate that there is specific complex formed between GPR39 and 5-$HT_{1A}$.

### 3.3. 5-$HT_{1A}$ and GPR39 heteromer analysis using co-immunopurification

We used co-immunopurification in order to provide further support to our FRET measurements on purified receptors. Heterocomplex formation among the three receptors (including $GalR_1$) was also tested using the same methodology (Fig. 2A). For heterodimerization experiments cells were co-transfected with equivalent amounts of 5-$HT_{1A}$-1D4 and GPR39-EYFP. The same amount of $GalR_1$ was added for heterotrimerization experiments. Immunopurification was carried out by immunoaffinity chromatography using the Rho-1D4 monoclonal antibody. $GalR_1$-1D4 and 5-$HT_{1A}$-1D4 were singly transfected, obtained separately and immunopurified to be used as immunoblot controls. Immunoblot analysis showed EYFP immunoreactive bands both in heterodimer and heterotrimer lanes, but with a different migration pattern. Both lanes showed two high molecular mass species as upper bands, but in the case of the dimer two lower bands could be detected and only one in the case of the trimer. No immunoreactive bands could be detected in control lanes as expected. When the inmunoblot was carried out using the anti-$GalR_1$ antibody, immunoreactive bands appeared in the $GalR_1$ control lane and in the trimer lane. Finally when the immunoblot was performed using Rho-1D4 antibody, we could detect signal in all lanes indicating that the immunopurification process worked successfully (Fig. 2A). These results indicated that we had purified the 5-$HT_{1A}$-GPR39 heteromer and the GPR39-5-$HT_{1A}$-$GalR_1$ complex. It can be noted that we could detect monomeric species for 5-$HT_{1A}$ and $GalR_1$ corresponding to the bands in the control lanes, but no further information could be derived from the other bands in our denaturing conditions. It is also noteworthy to indicate that in the trimer lane, the band intensity obtained for $GalR_1$ was lower than the intensity seen for the bands corresponding to the other two proteins. In other to discard the possible predominance of 5-$HT_{1A}$-GPR39, when compared to 5-$HT_{1A}$-GPR39-$GalR_1$, we analyzed the FRET curves between 5-$HT_{1A}$-ECFP and $GalR_1$-EYFP in the presence and in the absence of GPR39 at equivalent amounts.

### 3.4. Heterotrimer confirmation by FRET

In order to confirm the existence of a putative complex formed by our three working receptors, we measured FRET curves between 5-$HT_{1A}$ and $GalR_1$ receptors in the presence and in the absence of GPR39 and we compared them with those obtained using a negative control, also in the presence and absence of the same receptor (Fig. 2B). FRET was apparent in both samples and it had exactly the same shape. Two peaks could be clearly distinguished, one at 490 nm corresponding to ECFP (enhanced cyan fluorescent protein) and the other at 527 nm corresponding to EYFP, indicating that FRET was actually taking place. Thus, 5-$HT_{1A}$ maintained its capacity to interact with $GalR_1$ in the presence of GPR39. The same result was obtained for the negative control as expected (Fig. 2C).

### 3.5. SRE and NFκβ-RE luciferase reporter assays

Luciferase reporter assays were carried out with the purpose of analyzing differences in receptors signaling due to heteromer formation. Briefly, these assays are based on the fact that different GPCRs are able to activate different regulatory elements via interaction with specific G proteins populations. Therefore, activation of the different response elements can be an indirect measurement of receptor activation. In this particular case we measured SRE and NFκβ-RE activation. These two pathways were chosen because of the following reasons: on the one side, GPR39 signals via $G_q$, $G\alpha_{12/13}$ and $G\alpha_s$ after stimulation with zinc, and the activation of these G proteins leads to different pathways that

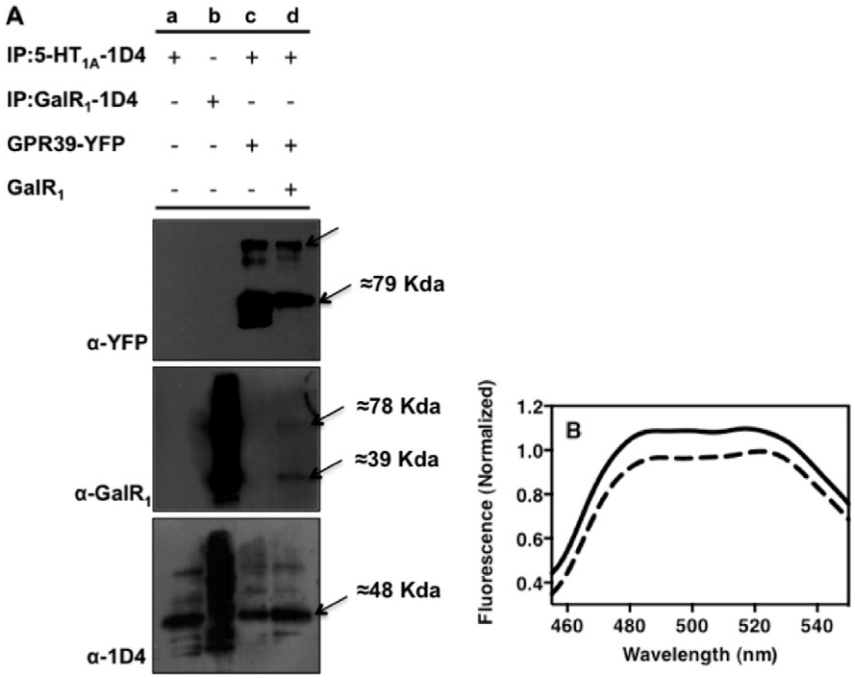


**Fig. 2.** Specific heterooligomer formation between 5-$HT_{1A}$-GPR39 and 5-$HT_{1A}$-GPR39-$GalR_1$. (A) Co-immunopurification immunoblots. Heterocomplexes were immunopurified by adding the 1D4 tag to 5-$HT_{1A}$ in combination with Rho-1D4 antibody coupled sepharose. Four lanes were loaded with the following samples: 5-$HT_{1A}$-1D4 (a), $GalR_1$-1D4 (b), 5-$HT_{1A}$-1D4 + GPR39-EYFP (c) and 5-$HT_{1A}$-1D4 + GPR39-EYFP + $GalR_1$ (d). Interaction between 5-$HT_{1A}$-1D4 and GPR39-EYFP is detected. Moreover, interaction between 5-$HT_{1A}$-1D4, GPR39-EYFP and $GalR_1$ is also detected. 5-$HT_{1A}$-1D4 and $GalR_1$-1D4 were used as control lanes. (B–C) FRET curves between 5-$HT_{1A}$-ECFP and $GalR_1$-EYFP. (B) Curves were obtained by cotransfection using two different conditions: 5-$HT_{1A}$-ECFP + $GalR_1$-EYFP (—) and 5-$HT_{1A}$-ECFP + $GalR_1$-EYFP + GPR39 (– – –) at equivalent concentrations. 5-$HT_{1A}$-ECFP + $GalR_1$-EYFP + GPR39 (– – –) data are nudged 0.1 points to avoid overlap. No differences between both curves are seen, meaning that 5-$HT_{1A}$-GPR39 is not the predominant form.

have as endpoints the activation of both response elements mentioned above, among others [12,23]. On the other side, 5-$HT_{1A}$ couples exclusively to $G_i/G_0$ that just activates SRE when 8-OH-DPAT is used as agonist [15]. This different behavior in receptor signaling is used to derive information about specific signaling of our studied receptors. The ability of our receptors to signal as mentioned and according to previously published data was experimentally tested (data not shown).

Heteromer exposure to 8-OH-DPAT or $ZnCl_2$ resulted in a significant SRE response compared to non-exposed samples ($p < 0.05$) (Fig. 3A) meaning that in the complex formed both receptors are active. Also, exposure to both compounds at the same time gave a significantly higher response compared to those exhibited by each one of the compounds on its own. These findings provide experimental evidence for additive signaling upon co-activation. But, if we take into account that the presence of zinc decreases 5-$HT_{1A}$ signaling ability, as it has been published [24] and we have experimentally tested (see Fig. S1), a plausible interpretation is that co-activation of both receptors increases GPR39 activity. In contrast, no activity could be detected after 8-OH-DPAT exposure alone (Fig. 3B) when analyzing NFκβ-RE signaling. This result is in agreement with the fact that 5-$HT_{1A}$ is not able to signal via NFκβ-RE and 8-OH-DPAT is not able to activate the GPR39 receptor. However, $ZnCl_2$ provided a significant signal increase because of GPR39 activation. Furthermore, upon co-activation of the two receptors the signal was significantly increased when compared to the signal corresponding to $ZnCl_2$ exposure alone. These results could mean that in the oligomeric state, and after co-activation of the two receptors, activation of 5-$HT_{1A}$ enhances GPR39 activity, as it is also suggested by SRE results, or alternatively changes 5-$HT_{1A}$ signaling possibilities.

Strikingly, the presence of $GalR_1$ blocks 5-$HT_{1A}$ and GPR39 signaling (Fig. 4). Thus, no SRE activation could be detected, in the presence of $GalR_1$, when cells were incubated with 8-OH-DPAT or $ZnCl_2$, whereas a significantly enhanced response could be observed when the sample was treated with gal (1–29) peptide (galanin full length peptide). Moreover, stimulation with all agonists together yielded the same response

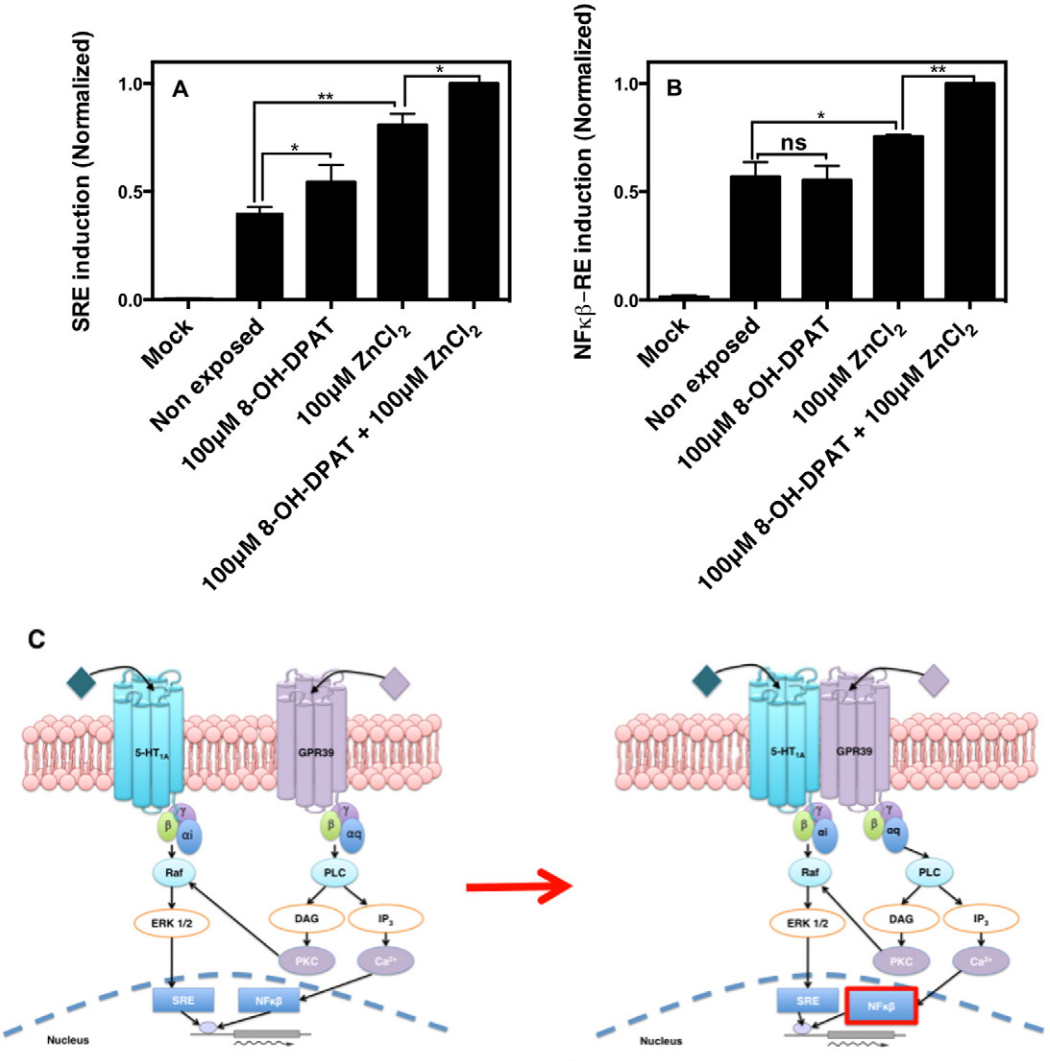


**Fig. 3.** SRE and NFκβ-RE luciferase reporter gene assay of signaling over the 5-$HT_{1A}$-GPR39 heteroreceptor complex. Mock transfected cells correspond to cells transfected without DNA and non-exposed cells to cells transfected but not agonist- stimulated. (A) SRE assay response after stimulation of co-transfected cells with 8-OH-DPAT, $ZnCl_2$ or both at the same time was measured. In the three cases we obtain signals significantly stronger compared to that of the control (non-exposed). Co-activation by agonists, at the same time, gives a significantly stronger signal than the ones obtained for single receptors demonstrating additive signaling. (B) NFκβ-RE response under the same conditions as earlier. In this case, 8-OH-DPAT activation of the complex is not observed, because 5-$HT_{1A}$ cannot act through NFκβ-RE. In contrast, activation mediated by both compounds exhibits a signal significantly higher compared to that obtained with $ZnCl_2$ activation alone. (A–B) The data represent the means ± SD of three independent experiments performed in triplicate. Statistical analyses were performed by one tailed paired t-test. Significantly different group comparisons are represented with *** ($P < 0.001$), ** ($P \leq 0.01$) and * ($P \leq 0.05$) and non-significantly different as ns ($P > 0.05$). (C) Schematic representation of signaling pattern changes between mono-homomeric forms and dimeric forms. Signaling through NFκβ-RE is reinforced due to 5-$HT_{1A}$-GPR39 heteromer formation. Adapted from [30,23]. Diamonds stand for receptors specific ligands.

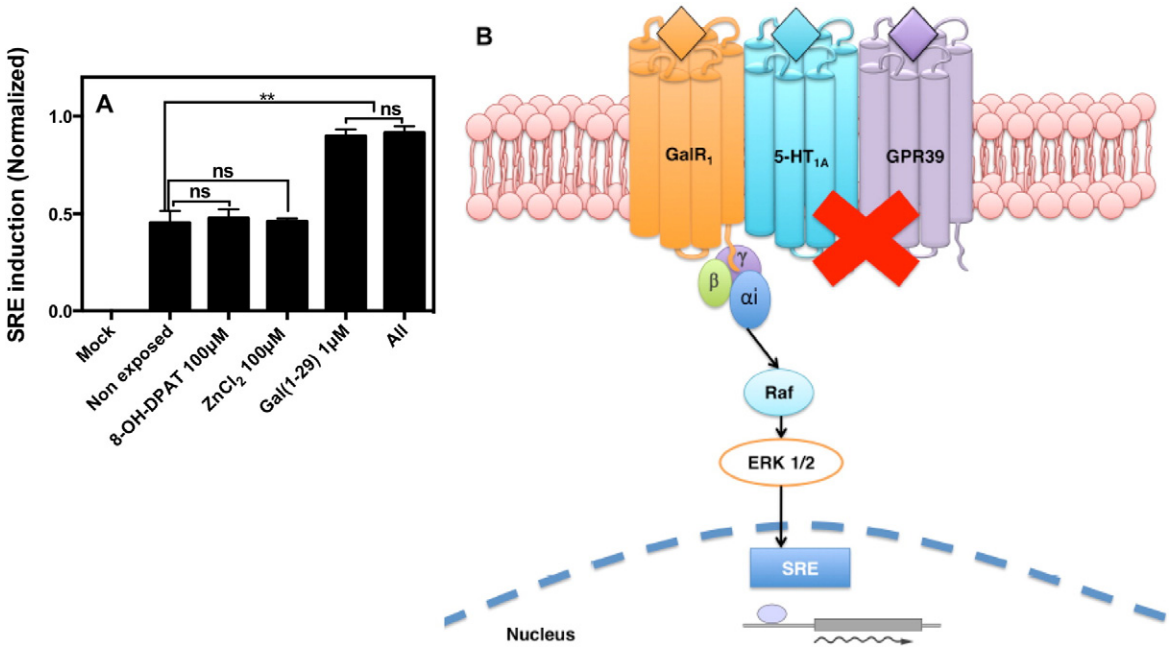


**Fig. 4.** SRE luciferase reporter gene assay of signaling over the 5-$HT_{1A}$-GPR39-$GalR_1$ complex. Mock transfected cells correspond to cells transfected without DNA and non-exposed cells to transfected but not agonist-stimulated cells. (A) SRE assay response was measured after stimulation of co-transfected cells with 8-OH-DPAT, $ZnCl_2$, gal (1–29) alone, or all combined. No response was obtained after stimulation with 8-OH-DPAT, or $ZnCl_2$, whereas a response could be detected after $GalR_1$ stimulation. Moreover, combined agonist activation of all receptors at the same time gives the same response as $GalR_1$ stimulation alone. The results indicate that the $GalR_1$ presence blocks SRE signaling of 5-$HT_{1A}$ and GPR39 protomers in $GalR_1$-5-$HT_{1A}$-GPR39 heteroreceptor complex. (A–B) The data represent the means ± SD of three independent experiments performed in triplicate. Statistical analyses were performed by one tailed paired t-test. Significantly different group comparisons are represented with *** ($P < 0.001$), ** ($P \le 0.01$) and * ($P \le 0.05$) and non-significantly different as ns ($P > 0.05$). (B) Schematic representation of the ERK 1/2-SRE signaling mechanism, found only in the $GalR_1$ protomer while this signaling pathway is blocked in the 5-$HT_{1A}$ and GPR39 protomers of this heteroreceptor complex. Other unknown pathways may be in operation at these protomers, adapted from [30,23]. Diamonds stand for receptors specific ligands.

as gal (1–29) alone. We conclude that when the three receptors interact, only $GalR_1$ is able to transduce signals trough the analyzed pathway.

## 4. Discussion

The relationship between 5-$HT_{1A}$-$GalR_1$ heteromer, depression and zinc has been previously postulated [14]. The potential linkage of GPR39 with zinc and its role in depression has also been proposed [4,17,25]. Also, a link between zinc and serotonin has been described, suggesting that serotonin levels can modulate GPR39 expression [26]. However, in spite of these potential associations, no experimental results have been reported on a direct relationship among the four studied elements (5-$HT_{1A}$-$GalR_1$, GPR39, zinc and depression). Our first characterization validated the co-localization of 5-$HT_{1A}$ and GPR39 in co-transfected cells and this work was continued by testing for heteromerization. In fact, heteromerization was demonstrated between the two receptors by means of FRET spectroscopy. 5-$HT_{1A}$ was found to interact with both $GalR_1$ and GPR39 suggesting a possible heteroreceptor complex formed by these three receptors. Such heterotrimer receptor complexes have previously been described for different GPCRs belonging to the same family [27,28].

The hypothesis was tested by using co-immunopurification. This method also allowed validating our previous FRET results indicating the existence of a 5-$HT_{1A}$-GPR39 heteromer. Immunoblots sustained the hypothesis of a heterotrimer built up of these three receptors. In the lane where the putative $GalR_1$-5-$HT_{1A}$-GPR39 complex had been loaded, signals were obtained when using the antibodies against each of the three proteins. However, in the lane where putative 5-$HT_{1A}$-GPR39 heteromers had been loaded, the signal was missing when using the anti-$GalR_1$ antibody. Also control lanes showed signals according to the antibody used. Furthermore, the signal corresponding to $GalR_1$ in the trimer lane was weaker compared to controls and signals corresponding to the other two proteins (Fig. 2A). As transfection had been done with equivalent concentrations of each cDNA, two explanations may be given for this result. On one side we can have the predominance of the 5-$HT_{1A}$-GPR39 heteromer versus the heterotrimer, and on the other side the possibility of an artifact, due to the high level of purified sample processed, cannot be ruled out. Alternatively, it is also possible that different receptor expression levels could be responsible for the different band intensities observed. FRET curves were recorded between 5-$HT_{1A}$ and $GalR_1$ in the presence or in the absence of GPR39. The presence of GPR39 did not modify FRET between the other two receptors, ruling out the possible predominance of 5-$HT_{1A}$-GPR39 heterodimer. If 5-$HT_{1A}$-GPR39 had prevailed over the $GalR_1$-5-$HT_{1A}$-GPR39 trimer, FRET may not have been observed. Two mechanisms can account for this behavior: the three receptors together form a stable complex or the predominance of one over the two different possible oligomers depends on cell type or specific physiological states.

The paradigm accepted for GPCRs oligomerization is that these receptors, as protomers, use this strategy in order to exponentially multiply their signaling capabilities [18]. Therefore, the main signaling characteristics of the heterodimer and the heterotrimer were further investigated.

Different signaling patterns were obtained for the heterodimer and the heterotrimer that were also different from the signaling pattern already published for the 5-$HT_{1A}$ and $GalR_1$ heteromer. The 5-$HT_{1A}$-$GalR_1$ heteromer shows trans-inhibition. Thus, when both receptors are ligand activated, the activity is exactly the same as that obtained when only one of the receptors is activated, either $GalR_1$ or 5-$HT_{1A}$ [15]. However, in the case of the GPR39-5-$HT_{1A}$-$GalR_1$ heterotrimer, $GalR_1$ acts as an antagonist blocking SRE signaling of the other two receptor protomers regardless of the fact that it is agonist activated or not. 5-$HT_{1A}$-GPR39 meanwhile shows additive increase in signaling along the SRE and NFκβ-RE pathways when both receptor protomers are agonist activated. This result is not consistent with monomer–homomer activity if we take into account that 5-$HT_{1A}$ is not able to

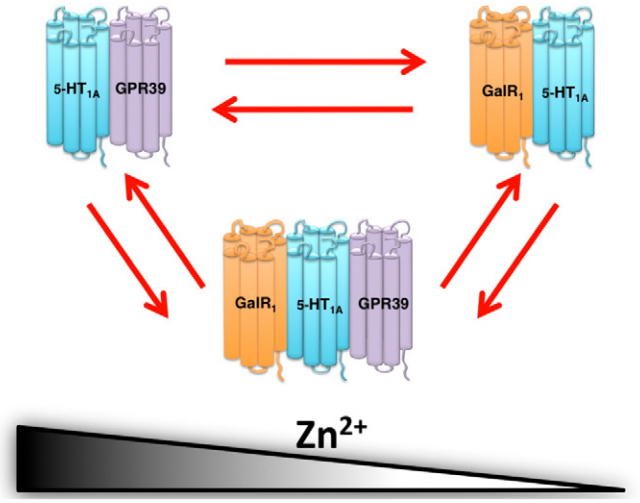


**Fig. 5.** Putative mechanism for zinc regulation of the balance of heteroreceptor complexes in which 5-$HT_{1A}$ protomers participate with GPR39 and/or $GalR_1$ protomers. High zinc concentrations counteract 5-$HT_{1A}$-$GalR_1$ heteromer formation which is replaced by the formation of 5-$HT_{1A}$-GPR39 heteromers. Instead, when zinc concentrations decrease, the 5-$HT_{1A}$-$GalR_1$ heteromer develops as the dominant form due to the decrease in GPR39 expression. In this process, the 5-$HT_{1A}$-GPR39-$GalR_1$ heteroreceptor complex participates as an intermediate form between the two heterodimers.

signal via NFκβ-RE. These findings suggest that heterodimerization confers new signaling opportunities to 5-$HT_{1A}$ and GPR39.

Our previous results strongly suggested that zinc disrupts the 5-$HT_{1A}$-$GalR_1$ heteromer [29]. Moreover, it was described that GPR39 knock-out mice exhibit depression-like behavior [25] and that zinc deficiency strongly decreased GPR39 expression, and gave depression-like behavior in rodents [12,13]. As a conclusion, the following scenario is introduced: zinc concentration regulates the oligomerization state between the three receptors $GalR_1$-5-$HT_{1A}$-GPR39 and this may have consequences in the regulation of the depressive behavior. At high zinc levels, 5-$HT_{1A}$ would no longer be able to interact with $GalR_1$, but could do so with GPR39, which is expressed at appropriate levels. In contrast, at zinc deficient levels, the predominant form would be 5-$HT_{1A}$-$GalR_1$ (vs 5-$HT_{1A}$-GPR39) since this heteroreceptor complex is not disrupted, and GPR39 levels might be low. The heterotrimer complex formed from the three receptors could be an intermediate between the two heterodimer complex states (Fig. 5). It is really difficult to associate a defined zinc concentration to each of the aforementioned states because the zinc concentration in a specific cell environment has not been clearly established. It depends on the cell type, and also on the specific state of the cell, varying from nM to mM concentrations [24]. However, according to previous reports [14,24] 50 μM should be considered a zinc concentration high enough to prevent depression.

Future work must strive to unravel the exact regulatory mechanism that zinc exerts on the oligomerization states among these distinct receptors (5-$HT_{1A}$-GPR39, 5-$HT_{1A}$-$GalR_1$ and $GalR_1$-5-$HT_{1A}$-GPR39) followed by validation of our results using human samples, and suitable techniques like PLA (proximity ligation assay). A full understanding of this process will increase the possibilities in the field of drug discovery and design in order to develop novel antidepressant drugs, involving e.g. specific divalent ligands for the heteroreceptor targets.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.09.003.

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